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Identification of the major soluble cuticular glycoprotein of lymphatic filarial nematode parasites (gp29) as a secretory homolog of glutathione peroxidase

(filariasis/*Brugia*/antioxidant/surface protein)

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ABSTRACT We have cloned and identified the major cuticular glycoprotein (gp29) of lymphatic filarial nematode parasites as a homolog of the antioxidant enzyme glutathione peroxidase. The derived amino acid sequence predicted a protein of 25.8 kDa, with an amino-terminal hydrophobic signal peptide and two sites for N-linked glycosylation, consistent with the documented properties of gp29. Transcription of a full-length cDNA in an SP65 vector and subsequent translation of the RNA in reticulocyte lysates *in vitro* generated a protein of 27 kDa, which was glycosylated upon the addition of pancreatic microsomal membranes. A postulated role for this secreted enzyme could be inhibition of the oxidative burst of leukocytes and neutralization of secondary products of lipid peroxidation, thus providing one explanation for the resistance of these parasites to immune effector mechanisms and their persistence in the mammalian host.

Lymphatic filariasis is a serious debilitating disease that affects some 90 million people worldwide (1). The causative agents, filarial nematodes of the genera *Brugia* and *Wuchereria*, are parasites that persist for long periods of time in the human lymphatic system. They achieve this longevity despite a vigorous host immune response and their apparent inability to undergo antigenic variation, implying a sustained resistance to or subversion of host immunity.

All nematodes are bounded by a cuticle, an extracellular matrix composed predominantly of collagens, and a highly cross-linked external envelope of proteins termed cuticlin (2, 3). In contrast to these conserved structural elements, the nematode cuticle exhibits an extremely restricted profile of soluble constituent proteins that may be species- or stage-specific (3, 4). The major protein of adult lymphatic filarial parasites defined by surface labeling is a 29-kDa glycoprotein (gp29) that appears to be expressed after infection of the mammalian host (5–11). Peptide mapping has shown that gp29 from *Brugia pahangi* and *Brugia malayi* are highly homologous (8), and the 29-kDa surface-labeled protein from *Brugia timori* (5) and *Wuchereria bancrofti* (6) probably represents the same molecule. In adult *B. malayi* gp29 is synthesized in the syncytial hypodermis, exported to the cuticle, and released into the external environment during *in vitro* culture (9, 10).

We report here the isolation and sequence of cDNAs that encode gp29 from *B. pahangi*,[†] which reveals that this cuticular protein is a homolog of the antioxidant enzyme glutathione peroxidase (GSHPx). A possible biological role for this enzyme in defense against immune-mediated cytotoxicity is discussed.

MATERIALS AND METHODS

Parasites. *B. malayi* were obtained from TRS laboratories, Athens, GA. Adult worms were recovered from the peritoneal cavities of jirds (*Meriones unguiculatus*) infected over 3 mo previously with 200 infective larvae and washed extensively with phosphate-buffered saline.

Labeling Procedures and Immunochemical Analysis. Adult *B. malayi* (mixed sex) were extrinsically labeled with Bolton–Hunter reagent and Iodo-Gen as described elsewhere (12). Parasites were also metabolically labeled with sodium [³⁵S]selenite at 0.25 mCi·ml⁻¹ (Amersham SCS-1; 1 Ci = 37 GBq) via *in vitro* culture for 24 hr in RPMI 1640 medium/2 mM glutamine/1% glucose/penicillin at 100 units·ml⁻¹/streptomycin at 100 µg·ml⁻¹. All labeled preparations were homogenized in phosphate-buffered saline/1.5% 1-octyl glucoside containing a mixture of protease inhibitors (12) and then centrifuged at 11,000 × *g*. Immunoprecipitation assays were done with 2.5 µl of antiserum in a total volume of 50 µl as described (12), resolved on 7–25% gradient polyacrylamide gels, and autoradiographed.

cDNA Isolation, Confirmation, and Sequence Determination. A cDNA library in bacteriophage λgt11 constructed from mRNA of mixed adult *B. pahangi* (13) was screened with a polyclonal antiserum to gp29 (9). Clones that reacted positively against gp29 in immunoprecipitation assays after affinity purification of antibody on plaque lysates (12) were selected for further analysis. cDNA 29.2 was excised from λgt11 with *Eco*RI and subcloned into pGEX-1 (14). The resultant protein, fused to glutathione *S*-transferase (GST), was expressed in *Escherichia coli* TG2 cells as described (14) and purified by preparative SDS/PAGE and electroelution. An antiserum was raised to the GST/29.2 fusion protein in a rabbit by a standard protocol and used in immunoprecipitation assays to confirm that the cDNA encoded gp29. Both cDNAs were subcloned into M13 vectors and sequenced on both strands via standard protocols of dideoxynucleotide chain termination.

Transcription and Translation *in Vitro*. The full-length cDNA (29.1) was excised from λgt11 with *Eco*RI and subcloned directly into pSP65 (15). The DNA template (5 µg) was transcribed with 10 units of SP6 polymerase (Boehringer Mannheim) in the presence of NTPs (each at 500 µM) at 40°C for 1 hr. The resultant RNA (0.5 µg) was translated in 20 µl of rabbit reticulocyte lysate (Amersham) with 100 µCi of [³⁵S]methionine for 1 hr at 30°C. Where indicated, 15 µl of canine microsomal membranes (Amersham) was added, and the reaction was done at 37°C for 1 hr. Products were digested

Abbreviations: GSHPx, glutathione peroxidase; GST, glutathione *S*-transferase.

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[†]The sequence reported in this paper has been deposited in the GenBank data base (accession no. X63365).

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with 6 units of N-glycanase (Genzyme) at 37°C for 16 hr as described (12).

RESULTS

Isolation of cDNA Clones. Approximately 10^5 cDNA clones were screened with a polyclonal antibody to gp29 (9), and 20 positives were plaque-purified. Of these clones, two cDNAs (29.1 and 29.2) elicited reactivity against gp29 in immunoprecipitation assays with antiserum that had been affinity purified on plaque lysates (data not shown). Verification that these cDNAs coded for gp29 was obtained by raising antiserum to the protein product expressed in *E. coli*. Thus, the cDNA insert of 29.2 was subcloned into pGEX-1 (see *Materials and Methods*), and a rabbit was immunized with the resultant fusion protein. This antibody specifically immunoprecipitated gp29 from adult worms that had been extrinsically labeled with iodine via either Bolton–Hunter reagent or an Iodo-Gen-catalyzed reaction (Fig. 1). Gp29 is the predominant protein labeled by the latter method (Fig. 1, IOD), whereas Bolton–Hunter reagent-mediated labeling is more pervasive (lane 1, BH). Control precipitations with the pre-labeled serum were negative (data not shown).

Properties of the Derived Protein Sequence. The nucleotide sequence of two overlapping cDNAs is shown in Fig. 2. The derived amino acid sequence predicts a protein with a mass of 25.8 kDa, two potential sites for N-linked glycosylation, and an amino-terminal hydrophobic stretch of 20 amino acids indicative of a signal peptide. A second substantial hydrophobic region exists from residues 65 to 79, but this does not show amphipathicity and is unlikely to represent a transmembrane segment. These data are consistent with the known properties of gp29—namely, (i) the native protein possesses two N-linked oligosaccharides, each with a relative mass of 2 kDa (8) and (ii) immunoelectron microscopic studies suggest that the protein is secreted from the underlying hypodermis (9, 10), and one would thus expect an appropriate signal peptide to be encoded.

The gp29 protein sequence was used to search the National Biomedical Research Foundation Genetics Computer Group data bases, and considerable similarity was observed to mammalian GSHPxs from diverse sources. Fig. 3 illustrates the homology of gp29 to GSHPxs from human liver and plasma (17, 18). A high degree of amino acid identity was seen

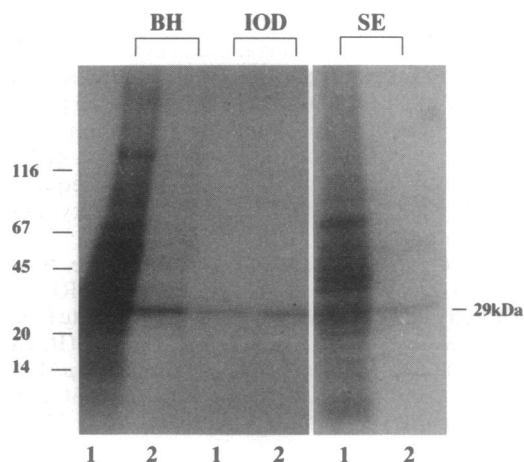


FIG. 1. Immunoprecipitation of gp29 from *B. malayi*. Lanes: 1, complete profile of proteins in adult parasites labeled with Bolton–Hunter reagent (BH), Iodo-Gen (IOD), and selenite (SE); 2, products of immunoprecipitation with a rabbit antiserum to a fusion protein expressed from cDNA 29.2. Proteins were resolved on a 7–25% gradient SDS/polyacrylamide gel; molecular size markers are given in kDa.

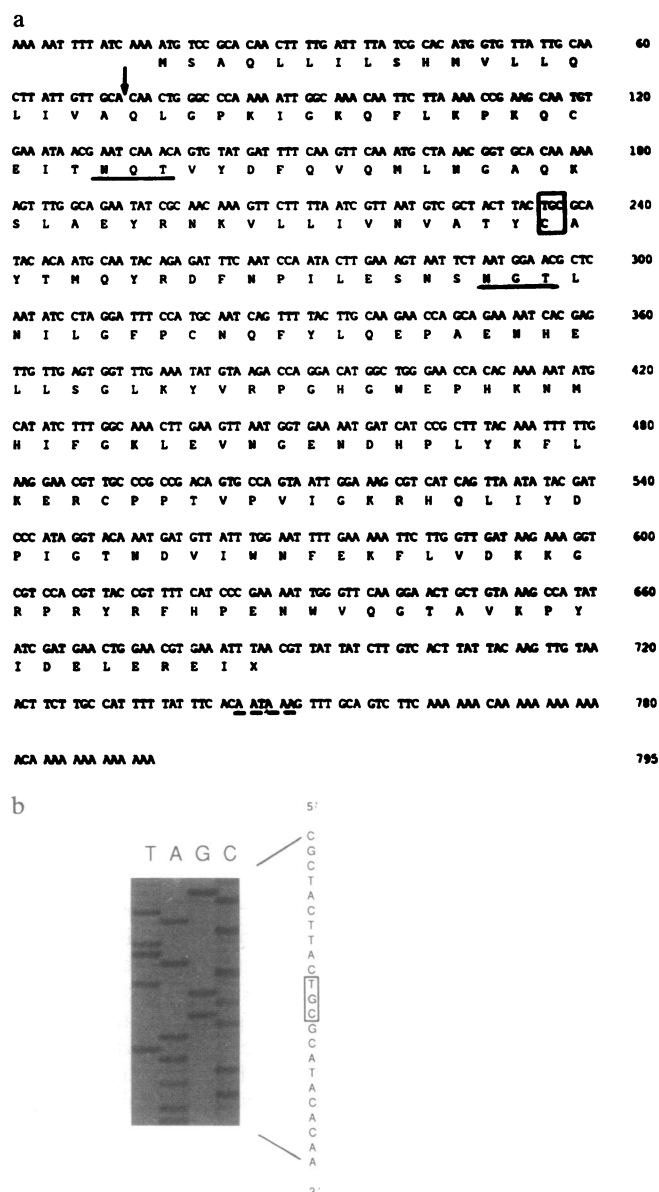
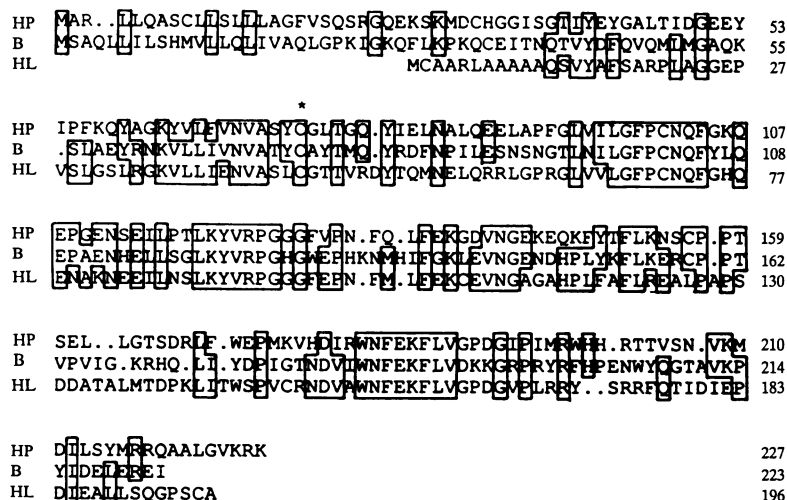


FIG. 2. Nucleotide and derived amino acid sequence of cDNAs encoding gp29. (a) Composite sequence of cDNA 29.1 (residue 1–684) and 29.2 (residue 478–795), determined by the dideoxynucleotide chain-termination method on both strands after subcloning into M13. Potential sites of N-linked glycosylation are underlined (—), and the polyadenylation signal sequence AATAAA is also indicated (---). The arrow marks a possible site for signal peptidase cleavage based on analysis of secretory proteins (16), and the position of the highly conserved selenocysteine of GSHPxs is boxed. (b) cDNA sequence around the site encoding selenocysteine. The UGC codon denoting cysteine is boxed.

to each enzyme (42% and 37%, respectively), although gp29 most closely resembles the plasma form in possessing an amino-terminal leader sequence. GSHPxs from plasma and erythrocyte cytosol exist in a tetrameric configuration (20), and some evidence suggests that gp29 from *B. pahangi* also adopts a tetrameric state (10).

The most significant difference between gp29 and mammalian GSHPxs lies in the codon that determines incorporation of the active-site selenocysteine in the latter enzymes (Figs. 2 and 3). In all selenoenzymes examined to date, selenocysteine is encoded by an in-frame UGA opal nonsense codon (21). In the cDNA for *B. pahangi* gp29, this UGA is substituted by UGC (Fig. 2a), a codon recognized by cysteinyl-tRNAs. Fig. 2b shows that this result is not from an

a



b

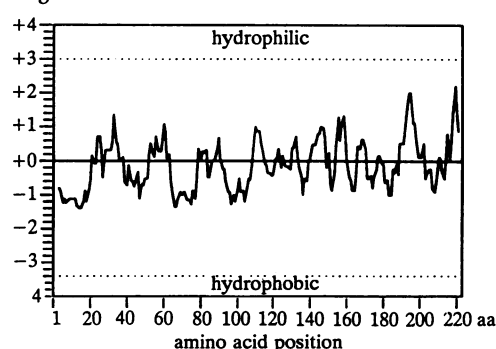


FIG. 3. (a) Alignment of the deduced amino acid sequence of gp29 from *B. pahangi* (B) with that of GSHPx from human liver (HL) (17) and plasma (HP) (18). Residues identical to the parasite sequence are boxed, and the position of the active site selenocysteine in the human GSHPx is marked with a star. (b) Hopp-Woods hydrophilicity plot (19) of gp29, determined in the Macintosh DNA INSPECTOR program and averaged over six residues.

error in interpretation of the sequencing gels. Moreover, we have isolated and sequenced the gp29 gene from genomic DNA of *B. pahangi*, *B. malayi*, and *W. bancrofti*, and they all show a UGC codon at this position (E.C. and M.E.S., unpublished data).

Selenium could be incorporated into gp29 by metabolic labeling of adult *B. malayi* with sodium [⁷⁵S]selenite over a 24-hr period of *in vitro* culture (Fig. 1). This result is not unambiguous, and additional experiments are required to determine definitively whether or not gp29 is a true selenoenzyme.

Transcription and Translation of cDNAs. To confirm that the cDNAs isolated for gp29 were representative of a secretory glycoprotein we subcloned clone Bp29.1 into the RNA expression vector pSP65 (15) and translated the derived transcript in a rabbit reticulocyte lysate with or without microsomal membranes. Fig. 4 shows that a protein with an estimated mass of 27 kDa was synthesized, and on addition of the microsomal membranes, a number of higher-molecular-size products were resolved. These products were removed by digestion with N-glycanase, producing an additional product of 25 kDa (Fig. 4). These data indicate that cDNA Bp29.1 encodes a protein that possesses a functional signal peptide that mediates translocation into the endoplasmic reticulum, followed by N-linked glycosylation. Thus gp29 appears to be processed like any standard secreted glycoprotein.

DISCUSSION

GSHPxs catalyze the reduction of hydroperoxides and are believed to protect membranes and other cellular components from oxidative damage (22). One obvious role for such an enzyme in helminth parasites would be defense against antibody-dependent cellular cytotoxicity directed by host leukocytes (23). This defense could be effected either directly, via removal of hydrogen peroxide generated by the cellular "respiratory burst," or secondarily by conversion of peroxidized fatty acids to their corresponding alcohols, thus limiting the chain reaction of lipid peroxidation. Relatively little is known about the *in vivo* mechanisms of immune-mediated killing of parasitic nematodes. Microfilariae (first-stage larvae of filarial nematodes) are susceptible to killing *in vitro* either via antibody-dependent cellular cytotoxicity or the direct application of isolated effector substances (23). In these assays, hydrogen peroxide proved the most toxic oxygen species toward microfilariae of *Onchocerca cervicalis* (24) and *Dirofilaria immitis* (25). In experiments on *B. malayi* microfilariae with purified eosinophil granule proteins, the most potent toxin on a molar basis was eosinophil peroxidase in conjunction with a hydrogen peroxide-generating system and a halide [i.e., a system capable of generating hypohalous acids (26)]. An active GSHPx that is secreted constitutively through the cuticle might, thus, help to protect the parasite against this form of immune attack.

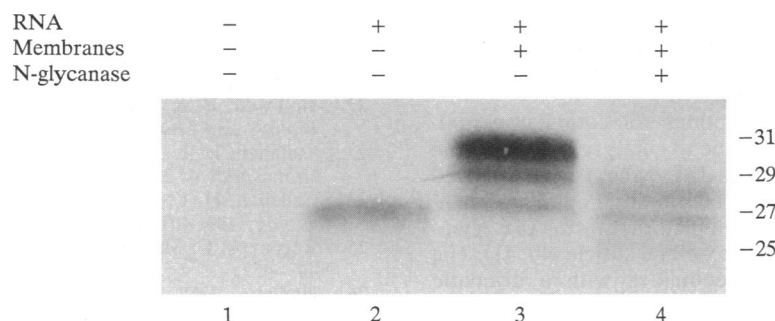


FIG. 4. *In vitro* translation of gp29. Translation products from a rabbit reticulocyte lysate resolved on a 15% SDS/polyacrylamide gel are shown with (+) or without (-) RNA and canine pancreatic microsomal membranes and after digestion with N-glycanase. Molecular sizes are given in kDa.

Consistent with this potential role, gp29 is expressed at negligible levels in mosquito-derived infective larvae, but after infection of the mammalian host, its synthesis is up-regulated, and the molecule is transported to the cuticle (11).

Recently, GSTs have been identified and cloned from *Schistosoma japonicum* (27) and *Schistosoma mansoni* (28, 29), trematode parasites of man. In the latter case, the enzyme is localized in the external tegument (29), and immunization of rats and mice with recombinant constructs results in a significant level of protection against challenge infection (28). GSTs could provide a "second-line" defense against oxidative attack via conjugation of electrophiles to glutathione and reduction of lipid hydroperoxides (22). Exposure of adult *S. mansoni* to glutathione-depleting agents renders them susceptible to killing by hydrogen peroxide (30), lending support to the concept of a protective role for GST and GSHPx.

The selenocysteine residue in GSHPxs is critical for the redox properties of the enzyme. Although the details of catalysis are not completely defined, the most probable reaction mechanism is a ping-pong process involving an initial oxidation of the enzyme followed by a two-step reduction of the oxidized enzyme by glutathione (31). Indeed, all selenoproteins that have a known function catalyze oxidation-reduction reactions (32). A suppressor tRNA charged with serine serves as the start-point for selenium biosynthesis in prokaryotes (33) and eukaryotes (34), although the reaction sequence may differ, as does the mRNA context required for suppression of termination (35, 36). If gp29 is a selenium-independent GSHPx, one would expect the velocity constants for the reaction with peroxides to be drastically reduced over an enzyme with a functional selenocysteine. Mutation of the selenocysteine of *E. coli* formate dehydrogenase to a cysteine reduced the catalytic efficiency of the enzyme by ≈ 380 -fold (37). A similar substitution in type 1 iodothyronine deiodinase from rats resulted in a protein with 10–20% of the activity of the wild-type enzyme (38), although a natural variant (type 11 iodothyronine deiodinase) exists that appears to possess cysteine in the catalytic site (39). The reason for the existence of a selenium-independent GSHPx in the cuticle of filarial parasites would, thus, be unclear, given the functional advantage of a selenol group over a thiol in terms of nucleophilic nature and catalytic potential.

An alternative possibility is that selenocysteine is formed posttranslationally in filariae. At first sight, the incorporation of radiolabeled selenite into gp29 shown in Fig. 1 suggests that this formation might be possible, but the chemical identity of the selenium moiety in the protein and the specificity of incorporation (there are four cysteine residues in the predicted sequence) is as yet unclear. Posttranslational formation would be extremely unusual, given that UGA codes for selenocysteine in all three lineages (eubacteria, archaeobacteria, and eukaryotes) (21). It is thus essential to determine the selenium content of native gp29 purified from parasite material and ideally sequence derivatized protein to resolve this paradox. With such limiting biological material, the latter task is quite formidable.

There are other possible functions for cuticular peroxidases in nematodes. One would be to catalyze the formation of cross-linking residues, such as dityrosine, trityrosine, and isotrityrosine, which have been identified in cuticular collagen and "cuticlin," a protein complex that forms the epicuticle, the structural external cortex of the cuticle (40, 41). The temporal expression of gp29 coincides with a dramatic growth phase in the life of the parasite, and an increased level of cross-linking of collagens and cuticlins may be necessary to maintain the high tensile strength of the cuticle. This postulated function may not be mutually exclusive with an antioxidant activity, however, and a highly cross-linked

external cortex may also serve to protect the parasite from immune attack.

Helminth parasites must survive for extended periods of time in their definitive host to ensure propagation of the next generation. In the absence of the capacity for antigenic variation, down-regulation or neutralization of the immune response most probably plays an essential role in parasite survival. Investigation of these defense mechanisms may reveal attractive targets for chemo- and immuno-prophylaxis.

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